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Protein–protein interaction studies provide evidence for electron transfer from ferredoxin to lipoic acid synthase in *Toxoplasma gondii*

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ABSTRACT

The only known redox system in the apicoplast, a plastid-like organelle of apicomplexan parasites, is ferredoxin and ferredoxin-associated reductase. Ferredoxin donates electrons to different enzymes, presumably including lipoate synthase (LipA), which is essential for fatty acid biosynthesis. We recombinantly expressed and characterized LipA from the protozoan parasite *Toxoplasma gondii*, generated LipA-specific antibodies and confirmed the apicoplast localization of LipA. Electron transfer from ferredoxin to LipA would require direct protein–protein interaction. Such a robust interaction between the two proteins was demonstrated in both yeast and bacterial two-hybrid systems. Taken together, our results provide strong evidence for a role of ferredoxin as an electron donor to LipA.

1. Introduction

Apicomplexa are intracellular protozoan parasites, which, like *Toxoplasma gondii*, the causative agent of human toxoplasmosis, possess a unique plastid-derived organelle called apicoplast. This non-photosynthetic secondary endosymbiont of an algal ancestor harbors several metabolic pathways, amongst which are fatty acid and isoprenoid biosynthesis [1]. Since both of these pathways in the apicoplast are of cyanobacterial ancestry and essential for the survival of the parasite, involved enzymes are of considerable interest as putative drug targets [2,3].

A protein redox system of central importance in the apicoplast consists of the small iron-sulfur cluster ([Fe-S]) containing plant-type ferredoxin (ptFd) and its associated NADPH-dependent reductase, plant-type ferredoxin-NADP$^+$-reductase (ptFNR) [1,4,5]. Reduced ptFd can donate electrons via protein–protein interaction to acceptor proteins involved in various metabolic pathways [6]. In the apicoplast so far the only known ptFd-interacting protein is LytB, which catalyzes the second-last step in the synthesis of the isoprenoid precursors [7,8]. However, based on experimental work in other cellular systems it is very likely that ptFd is also an electron donor for other apicoplast-resident pathways, like [Fe-S] or fatty acid synthesis (FAS), respectively [1,4].

We previously reported the cloning of lipoate synthase (LipA in *Escherichia coli*) from *T. gondii* (TgLipA) that was targeted to the apicoplast as shown by GFP-tagging of N-terminal parts of the protein [9]. Lipoic acid (LA) is an essential co-factor for the majority of prokaryotic and eukaryotic organisms [10] since the post-translational modification of several 2-oxoacid dehydrogenase subunits with LA is instrumental for catalysis [11]. One of these enzymes is pyruvate dehydrogenase (PDH), an essential enzyme complex required for the generation of the FAS precursor acetyl-CoA also in the apicoplast [1]. To start LA synthesis an octanoyl group from octanoyl-ACP is enzymatically attached to a specific lysine side chain in what is to become a lipoylated protein. Then the insertion of two sulfur atoms into the carbon chain of the protein-bound octanoate is performed by LipA (see Fig. S1) [12,13]. A chemically similar reaction is carried out by biotin synthase [14]. Both proteins are members of the so-called "radical SAM" superfamily [15,16]. They depend on S-adenosyl methionine (SAM) for their radical-based catalysis of the different reactions, and an electron transfer system required for radical generation. All enzymes of this class are [Fe-S]-containing proteins. LipA is thought to contain two [4Fe-4S], of which one donates the two sulfurs to the octanoate side chain during catalysis [16,17].

Abbreviations: bGal, β-galacosidase; FAS, fatty acid synthesis; [Fe-S], iron-sulfur cluster; LipA, lipoate synthase; MU, Miller units; MW, molecular weight; PPI, protein–protein interactions; ptFd, plant-type ferredoxin; ptFNR, plant-type ferredoxin-NADP$^+$-reductase; SAM, S-adenosyl methionine; RTH, reverse two-hybrid; YTH, yeast two-hybrid

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While it is known from bacteria that the generation of both, biotin and lipoic acid, acquire electrons from the redox protein flavodoxin [18,19], the electron donor in the apicoplast is currently not known (Apicomplexa lack flavodoxin), but the most likely candidate is the ptFd/FNR redox system [1,4].

2. Material and methods

2.1. Recombinant TgLipA expression, purification and analysis

The TgLipA-containing expression vector pS1-TgLipA as well as expression conditions have been described previously [9]. pS1-TgLipA encodes an N-terminally 6His-tagged TgLipA protein devoid of its predicted N-terminal bipartite targeting domain of 179 aa, which are sufficient to target GFP to the apicoplast [9]. To increase [Fe-S] assembly on TgLipA plasmid pMK400H lacking LipAH was co-expressed [20]. This plasmid contains the *iscSUA* and *hscBAfdx* gene clusters and leads to greatly improved [Fe-S] incorporation into recombinant proteins. Purification of recombinant TgLipA by Ni-NTA affinity chromatography in 300 mM NaCl, 50 mM Na-phosphate pH 8.0, 20 mM imidazole was performed as detailed by the supplier of the resin (Qiagen) using an Äkta Purifier FPLC system (GE Healthcare). Elution from the column was performed in the same buffer containing 250 mM imidazole. The brown eluate was subjected to gel filtration chromatography on a PD-10 column (GE Healthcare) for buffer exchange to PBS. The resulting protein solution was stored in small aliquots at −70 °C. All steps were performed under aerobic conditions.

SDS–PAGE and Western blot analysis of *E. coli* lysates or purified protein was done as described previously [5]. Gel filtration chromatography of purified recTgLipA on a Superdex 200 10/300 GL column at a flow rate of 0.7 ml/min in PBS was performed under aerobic conditions using the FPLC system. Calibration of the Superdex 200 column was performed with MW standards ferritin, catalase, aldolase, albumin and chymotrypsin (GE Healthcare) under the same conditions. One ml fractions were collected and 500 µl of each were acetone precipitated overnight at −20 °C. Pellets were solubilized in SDS–PAGE sample buffer and run on a 10% SDS-PAG.

DTT treatment and reconstitution of TgLipA were done according to [18]. After 10 min on ice in the presence of 5 mM DTT 800 µM $FeCl_3$ were added to 100 µM TgLipA in 200 µl PBS, followed by the same amount of Na_2S. The mixture was kept on ice for 3 h before it was centrifuged at 15 000 rpm for 15 min at 4 °C. 40 µl of the supernatant were then analyzed by gel filtration on the Superdex 75 column as described above. The column was calibrated with ribonuclease A, chymotrypsinogen A, ovalbumin and albumin (GE Healthcare).

2.2. Yeast two-hybrid system

The yeast two-hybrid system (YTH) based on B42AD and LexA interacting domains [21] was essentially used in the same way as previously described by us for the interaction analysis of TgFd and TgFNR [22,23], following the protocols of the supplier of the YTH system (Origene), with the exception that in the respective plasmids either TgFd or TgFNR were replaced by TgLipA, which was re-cloned from pS1-TgLipA (see above) into the yeast vectors (see Suppl. Material). All newly assembled parts of the constructs were sequenced.

2.3. Bacterial reverse two-hybrid system

The bacterial reverse two-hybrid system (RTH) used is that described initially by Di Lallo et al. [24] and modified by Horswill et al. [25]. For details on construction of plasmids and strains see Fig. S2. *E. coli* cultures were maintained in LB broth. DNA manipulations were performed using standard techniques unless otherwise indicated, and plasmids were transformed into *E. coli* PIR1 cells (Invitrogen) by electroporation with a BioRad Genepulser II. All DNA sequencing was performed at the sequencing facility of the Robert Koch-Institute.

Antibiotics were provided at the following concentrations (lower for chromosomal markers): tetracycline 10 µg/ml, spectinomycin 100 (35) µg/ml, kanamycin 50 (10) µg/ml, gentamicin 15 (5) µg/ml, ampicillin 100 (50) µg/ml. MOPS minimal medium [26] supplemented with 0.1% glucose and 0.132 mM K_2HPO_4 was used for β-galactosidase assays.

2.4. Kinetic β-galactosidase assay

The following procedure was based on the protocol of Thibodeau et al. [27]. Cultures of *E. coli* strains containing integrated reporter and repressor constructs were grown in MOPS medium overnight at 37 °C ± IPTG induction (100 µM). 150 µl of culture or appropriate amounts with an OD_{600} of 0.15 (measured with a Tecan M200 Pro microplate reader) were diluted into 3 ml MOPS medium and again grown overnight at 37 °C. 2 ml of culture or appropriate amounts with an OD_{600} of 0.14 were harvested by centrifugation and resuspended in 100 µl MOPS medium. Cell lysis reagent (0.05 M HEPES pH 7.5, 1% Triton X-100, 0.4% Tergitol NP-9, 3.8×10^{-5} M polymyxin B) was added and incubated for 15 min at room temperature.

15 µl of each lysate were placed as duplicates into individual wells and a mix of 135 µl Z buffer (0.06 M $Na_2HPO_4{*}7H_2O$, 0.04 M $NaH_2PO_4{*}H_20$, 0.01 M KCl, 0.001 M $MgSO_4{*}7H_2O$, containing 2.7 µl 2-mercaptoethanol for every 1 ml of Z buffer) and 30 µl ortho-nitrophenyl β-D-galactoside solution (4 mg/ml) was added. The assay was performed in the microplate reader set at 28 °C, read at 415 nm, and Miller units (MU) were calculated as described [27].

2.6. Generation of anti-TgLipA antiserum

The generation of anti-TgLipA antiserum in a rabbit was done as previously described [5] using purified recombinant protein. To obtain monospecific polyclonal antibodies the resulting serum was purified on 6His-TgLipA immobilized on a Co^{2+} TALON metal affinity resin (Clontech) as described previously [28,29]. The eluted antibodies were then stored in PBS at −20 °C.

2.7. Parasite and host cell culture and immunofluorescence assay

T. gondii transgenic strain RHβ1 expressing *E. coli* β-galactosidase [30] was maintained in confluent human foreskin fibroblasts (HFF, BJ-5ta; ATCC CRL4001) using Dulbecco's modified Eagle's medium with high glucose and stable glutamine (DMEM; PAA) supplemented with 10% fetal calf serum (Gibco) and antibiotics. Tachyzoites grown in HFF on coverslips in a 24 well plate for 24 h were fixed for 20 min in 4% paraformaldehyde, permeabilized 20 min with 0.25% Triton X100, and stained using the rabbit anti-LipA polyclonal antibodies (dilution 1:30) and a Cy3-coupled secondary goat anti-rabbit antibody (Jackson ImmunoResearch, diluted 1:300). For localization of biotin-containing proteins in the parasite [31] Alexa 488-tagged streptavidin (Invitrogen, 1:5000) was used. DNA was stained with 4′,6-diamidin-2-phenylindol (Sigma–Aldrich, IL, USA, 1 ng/µl). Samples were visualized using a Zeiss Axio Imager Z1/Apotome microscope. Images were acquired with a Zeiss AxioCam MRm camera using AxioVision software, and processed using equal linear adjustments for all samples. Image analysis for co-localizations were done using ImageJ 1.47 m

and plugins "Colocalization Finder" and "Blend Images" (http://imagej.nih.gov/ij/plugins/index.html).

2.8. Statistical analyses

Statistical significance of the difference between groups was calculated with Prism5 software (GraphPad), using an unpaired two-tailed *t*-test.

3. Results and discussion

3.1. Production and characterization of recombinant TgLipA

TgLipA was expressed in *E. coli* as a 6His-tagged protein. Expression was robust (ca. 3 mg/l) and yielded a dark brown cell pellet. A single round of affinity chromatography purification on Ni-NTA resin under aerobic conditions resulted in high yields of a soluble, slightly brownish protein that showed a MW of 62.6kDa on a calibrated analytical Superdex 200 gel filtration column (Fig. 1A). A second, more prominent peak of 133kDa, indicative of a dimer, as described earlier for LipA from *E. coli* [32,33] was also observed. Several fractions from this gel filtration column, including those from the left shoulder >133kDa, and a peak larger than 1700kDa (presumably aggregates), where analyzed by SDS–PAGE. All contained a single protein of ca. 42–43kDa, as judged by SDS–PAGE (see Fig. 1A inset), with only traces of other contaminating proteins (Fig. 1A inset). This molecular mass is in good agreement with the calculated MW of 6His-TgLipA (41.9kDa). Control experiments in a Δfdx *E. coli* strain ruled out that the observed 11kDa peak contains bacterial ferredoxin (data not shown).

Interestingly, when we subjected TgLipA to DTT treatment prior to gel filtration and analyzed it on a Superdex 75 column to achieve better separation of protein species <70kDa we observed only a major peak at 46kDa (Fig. 1B), close to the 41.9kDa theoretical MW of TgLipA, and a minor peak at 74kDa. The 46kDa protein showed only minimal absorption at 465 nm (used to detect the presence of $[2Fe\text{-}2S]^{2+}$ in monomeric *E. coli* LipA (EcLipA; [33]) and is thus presumably a cluster-free apo-form of TgLipA. After reconstitution of the [Fe-S] of DTT-treated TgLipA by incubation for 3 h in the presence of Na_2S and $FeCl_3$ followed by immediate gel filtration (both under aerobic conditions) peaks of 60.3 and >94kDa appeared, both with clear signals at 465 nm (Fig. 1B). The latter MW cannot be assigned correctly on this column because its optimal separation range is only up to 70kDa, but as shown on the Superdex 200 in Fig. S3, it is the dimeric form of TgLipA. Since after reconstitution the monomeric form of TgLipA is more prominent over the dimeric form we conclude that the latter predominantly contains partially or fully oxidized [Fe-S] whereas they are intact in the monomeric form. It is well described in the literature that partially iron-loaded or not fully reconstituted [Fe-S] proteins even under strictly anaerobic conditions form multimers/dimers instead of monomers [34–37].

A monomeric form of TgLipA is also consistent with the recently reported three-dimensional structure of LipA2 from the cyanobacterium *Thermosynechococcus elongatus*, which presents as a monomer in the crystals [17]. The described dimeric form of EcLipA [32,33] seems to be due to the exquisite lability of the [4Fe-4S] when not purified under very strict anaerobic conditions [38]. Given the high sequence identity and similarity (46% and 59.5%; Fig. S4A) in the homologous part between both proteins, including the serine that serves as a newly described ligand to the auxiliary cluster [17], we modeled the structure of TgLipA based on an alignment of aa 6–288 of *T. elongatus* LipA2. The result shows a high degree of structural similarity over the entire aligned sequence (Fig. S4B).

The size differences of the apo-form of TgLipA compared to the monomeric form during gel filtration is quite substantial (15–20kDa). The crystal structure of TeLipA2 shows an 18 Å deep channel into which the octanoyl moiety could be modeled, packed between SAM and the auxiliary [Fe-S] [17]. In the apo-form, when the clusters are removed, the substrate-binding channel might collapse, leading to the observed size difference. An interesting hypothesis would be that this compact form is the one recognized by the [Fe-S] synthesis/repair machinery.

However, TgLipA is also considerably longer at both termini (38 aa at the N-terminus – not considering the additional 179 aa for apicoplast targeting – and 45 aa at the C-terminus). Thus, alternatively or in addition to the explanation above the structure of these regions might contribute to the larger MW observed in gel filtration experiments. We analyzed the TgLipA sequence used

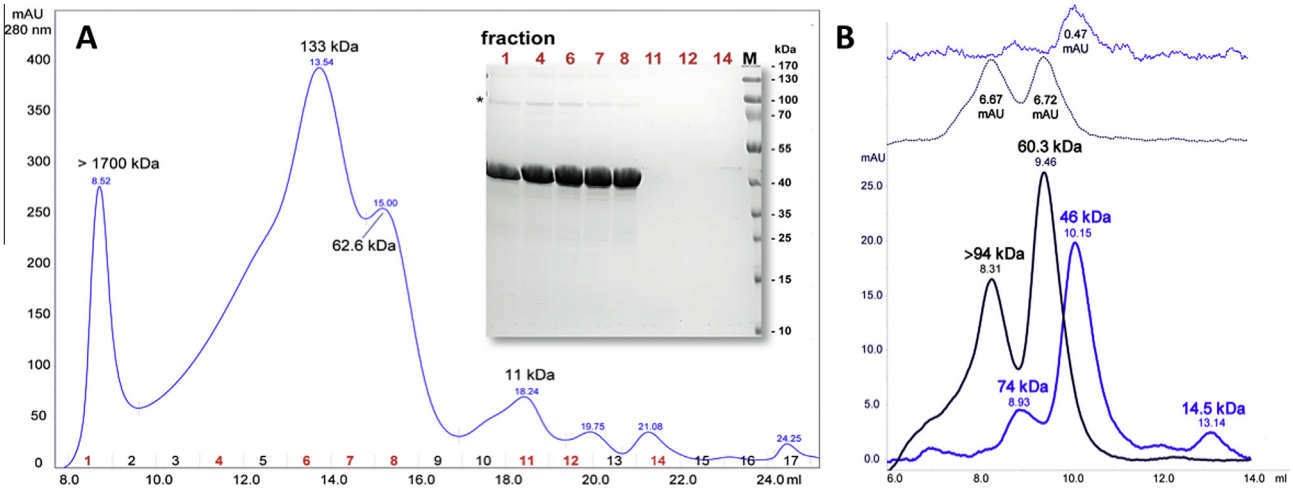

Fig. 1. Gel filtration and SDS–PAGE analysis of purified recombinant TgLipA. (A) 2 mg of purified recombinant TgLipA in 500 µl PBS were chromatographed over a Superdex 200 gel filtration column and developed with 35 ml PBS (chromatogram for 25 ml is shown). (A inset) One ml fractions of (A) were sampled and 1/2 of some fractions (numbers above *X*-axis) were subsequently analyzed by 10% SDS–PAGE. *Denotes a weak band that reacted with anti-6His antibodies on a Western blot (not shown) and thus most likely constitutes dimeric TgLipA. (B) DTT-treated (blue line) and $FeCl_3/Na_2S$-reconstituted (black line) TgLipA was chromatographed under identical conditions on a Superdex 75 gel filtration column. The top chromatograms (dotted lines) show the 465 nm recordings (at different scales) whereas the bottom solid lines are the recordings at 280 nm. Note the differences in peak heights in mAU at 465 nm given in numbers between the black and blue lines. Due to the size limitation of the column the size of the extrapolated >94kDa peak is inaccurate (see Fig. S3 for additional analysis). See also text for details.

for expression by a number of software tools intended to detect intrinsically disordered structures (see Fig. S5). Such sequences are known to significantly alter the stokes radius of proteins in gel filtration experiments [39]. Almost all the tested algorithms indicate that the N-terminal 50–70 aa are disordered, and several predict this also for the C-terminus. The same analyses for EcLipA indicate much less disordered structures at the termini, consistent with a less pronounced difference between observed and calculated MW (7kDa; [32]).

Since this is the first recombinant LipA enzyme from eukaryotes described in the literature the relevance of these observations for function or protein interaction need to be determined.

3.2. Antibodies directed against TgLipA confirm its apicoplast localization

In our previously published experiments we had used only the N-terminal 179 aa of TgLipA fused to GFP in combination with a heterologous promoter for our genetic targeting constructs to reveal the localization of TgLipA [9]. For many nuclear-encoded, apicoplast-targeted proteins this approach is well established and leads to reliable results. However, the N-terminus of TgLipA is unusual since it is very long and none of the current computer algorithms predicts a signal peptide for it (data not shown), which is a prerequisite for most proteins to be imported into the apicoplast [40]. Moreover, expression from non-homologous promoters can in some cases lead to mislocalization [41]. Therefore, we wished to formally prove the apicoplast localization of TgLipA using polyclonal antibodies raised against the purified recombinant protein. The resultant antiserum was purified on immobilized 6His-TgLipA (see Methods) and the eluted monospecific polyclonal antibodies used for indirect immunofluorescence assays on HFF infected with tachyzoites. Using both, biotin attached to apicoplast-resident acetyl-CoA carboxylase as well as organellar DNA as two independent markers for the apicoplast [31] we found that the anti-TgLipA antibodies also decorated this organelle (Fig. 2), confirming that TgLipA is exclusively apicoplast-localized.

3.3. TgLipA interacts with ferredoxin in a yeast two-hybrid system

Electron transfer between ptFd and any acceptor protein requires physical contact between both proteins. Consequently, interaction of ptFd with a protein can be taken as strong evidence that this protein is a likely acceptor for ptFd-derived electrons. It was shown previously that in *Plasmodium falciparum* ptFd provides electrons to the last enzyme of the apicoplast-resident isoprenoid synthesis pathway, LytB, by physical interaction [7]. To ask whether TgLipA interacts with TgFd in a cellular environment we focused on in vivo interaction experiments with the YTH system for two main reasons. Any in vitro assay for LipA requires very strict anaerobic conditions (see above), necessitating equipment available only to few specialized labs. In addition, LipA is present in cells only in minute amounts [42], making proteomic approaches with parasite material barely feasible.

Fig. 3 reports interactions as β-galacosidase (bGal) activity in Miller units (MU). As can be seen, TgLipA and TgFNR showed only little bGal activity above background, indicating no specific protein–protein interaction (PPI). In contrast, TgLipA interacted equally well with either *P. falciparum* or *T. gondii* ptFd. TgLipA interacts specifically with apicomplexan ptFd in a similar robust manner in this system when compared to the well-characterized interaction of TgFd with TgFNR, which lies in the low nanomolar range [22,23]. The interaction of a cyanobacterial ferredoxin I (PetF from *T. elongatus*, 55.7% identical to TgFd) and the second last enzyme of the isoprenoid synthesis pathway, GcpE, was previously also demonstrated in a YTH system [43], validating this approach for defining ptFd-interacting proteins.

3.4. TgLipA and TgFd also interact in the reducing cytosolic environment of E. coli

Since the apicoplast is considered to be a reducing environment [44] we wished to verify the apparent TgLipA–TgFd interaction in the reducing cytosol of *E. coli*. For this we chose a bacterial reverse two-hybrid (RTH) system that is based on a chimeric operator formed by the two half sites of the phage P22 and phage 434 operators [24]. Fusion of the two short phage proteins at the N-terminus with the proteins of interest lead to a functional repressor in case the two heterologous proteins interact, rather than a transcriptional activator, as is the case in the yeast-based system. Consequently, the readout for PPI is diminished reporter activity upon expression of the protein pair (arranged in an operon driven by an IPTG-inducible lac promoter) compared to the non-induced cultures [25]. Using this RTH system we evaluated PPI of TgFd with

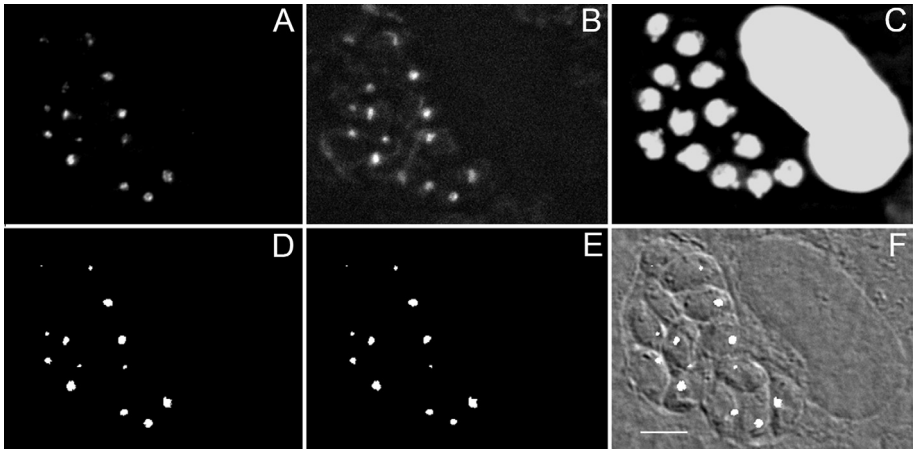

Fig. 2. Detection of LipA in the apicoplast of tachyzoites by immunofluorescence microscopy. (A) Tachyzoites within infected HFF-cells were decorated with polyclonal anti-TgLipA rabbit antibodies. (B) *T. gondii* possesses three biotin-containing proteins, two acetyl-CoA carboxylases, localized in the apicoplast and cytosol, respectively, and pyruvate carboxylase in the mitochondria [31]. Consequently, incubation with streptavidin-Alexa 488 reveals prominent staining of the apicoplast and additional much weaker signals in the mitochondrion. (C) DNA staining with DAPI also allows visualization of the apicoplast genome. Applying the 'Colocalization Finder' plugin of ImageJ on (A) with (B) results in (D), and on (A) with (C) results in (E). White pixels demonstrate that in both cases TgLipA co-localizes with the respective apicoplast markers. Merger of co-localization areas of (D) with a DIC image (F). Scale bar, 5 μm.

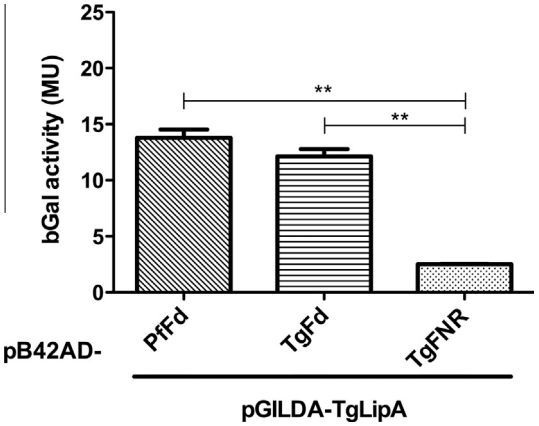

Fig. 3. Interaction of TgLipA with TgFd in a YTH system. Yeast strains containing the indicated plasmids (pB42AD and pGILDA) were grown under inducing conditions (+galactose), and bGal activity was determined. TgFNR served as non-interacting control for the statistical analysis of both pGILDA sets (interacting partners for TgLipA and TgFd, respectively). $^{**}P < 0.01$ ($n = 2$).

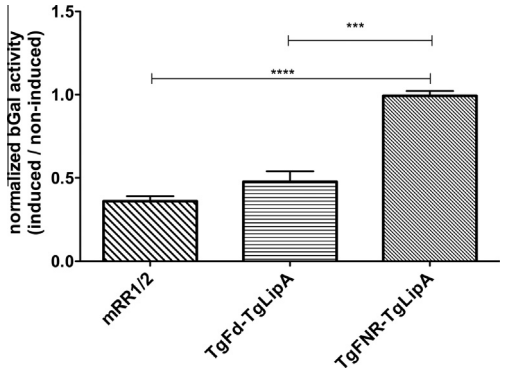

Fig. 4. Interaction of TgLipA with TgFd in a bacterial RTH system. Bacterial cultures containing the indicated gene pairs were grown under inducing or non-inducing conditions and their bGal activity determined. The ratio of the mean MU from 3 independent experiments from induced (+IPTG = possibly repressed) and non-induced (−IPTG = non-repressed) cultures was calculated. A ratio of 1 means no interaction (no repression), whereas 0 reflects 100% repression and thus a very strong interaction. As non-interacting control served TgFNR–TgLipA. $^{****}P < 0.0001$; $^{***}P < 0.0009$.

TgLipA. A fusion of TgLipA and TgFNR served as negative control, and the small and large subunits of murine ribonucleotide reductase (mRR1/2) as positive controls [25], respectively.

As can be seen in Fig. 4 induction of expression by IPTG led to repression of bGal activity to ⩽40% of the activity of the non-induced culture in the case of the positive control mRR1/2, whereas there was no repression for TgLipA-TgFNRwt, indicating good discriminatory power of the system to reveal specific PPI. When TgFd was evaluated with TgLipA, repression of more than 50% was observed in three independent experiments, indicating that these two proteins interact also in a reducing environment and in a different cellular context. Taken together, TgFd and TgLipA specifically interact with each other also in a bacterial RTH system.

4. Conclusions

Collectively, to our knowledge this is the first experimental evidence that a plant-like ferredoxin and lipoate synthase from an eukaryote directly interact with each other. It is very likely that this leads to subsequent electron flow from NADPH via TgFNR to TgFd to TgLipA for the production of SAM radicals and subsequent sulfur insertion into octanoylated PDH-E2 (Fig. S1). Our data define a further acceptor enzyme for this important redox system, and since lipoylated PDH is an essential prerequisite for fatty acid biosynthesis [45,46], disruption of ferredoxin's ability to donate electrons to LipA could be exploited as a potential intervention strategy against *T. gondii*. In this respect, the bacterial RTH system together with genetically encoded peptides will be a promising approach to search for such pharmacophores [25].

In plant mitochondria the interaction of biotin synthase with the mitochondrial ferredoxin redox system has been reported [47]. Since biotin synthase and LipA share the same overall chemistry for sulfur insertion, and in plants (unlike in Apicomplexa) lipoate synthesis also occurs in the mitochondria [48] our data suggest that mitochondrial ferredoxin could also serve as electron donor for lipoate synthase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.11.020.

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